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<p>We have designed metal ion biosensors through the template-based incorporation of metal-binding sites into an antibody scaffold. We constructed a high-resolution three-dimensional model of the variable domain of the catalytic antibody NPN43C9 based on our antibody structural database. By transplanting metal-binding templates from metalloenzymes into the antibody structural model, we identified potential metal-binding sites within the antigen-binding pocket, where bound metal could be detected by spectral changes of the antibody or of bound antigen analogs. Three distinct metal-binding sites, one with ligands in the VL domain, one with ligands in the VH heavy domain, and one with ligands in both domains, were made collaboratively in the Benkovic laboratory by site-directed mutagenesis and shown spectroscopically to bind metal ions as predicted. These results demonstrated the success of our design approach, the versatility of the antibody structure for metalloprotein design, and the validity of the three-dimensional model. One site shows selectivity for Cu(II) over Zn(II), while the other two sites display a more unusual preference for Zn(II) over Cu(II). Thus, we have designed metal-binding sites having different metal specificities into a single antibody. A panel of such mutants proteins, based on a single antibody, could act as a biosensor for multiple metal ions.</p>			
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FINAL TECHNICAL REPORT

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PRINCIPAL INVESTIGATOR: Elizabeth D. Getzoff

INSTITUTION: The Scripps Research Institute

GRANT TITLE: Design of Metal-Detecting Antibodies

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OBJECTIVE: To analyze the geometric and functional group requirements of protein metal-binding sites for the design of proteins with predetermined metal-binding specificities. Antibodies were chosen as the basis for template-based metalloprotein design because they can be readily produced and easily incorporated into a variety of detection systems.

ACCOMPLISHMENTS:

The Three-Dimensional Model of the NPN43C9 Antibody. A model of the variable (Fv) domain of the catalytic antibody NPN43C9 was constructed based on our antibody structural database (ASD). The ASD (1) is a computer graphics database of the known crystallographic structures of antibodies, containing superimposed variable light (VL), and variable heavy (VH) antibody chains, and the Fv domains. The model of NPN43C9 revealed that the positively charged side chain of VL residue Arg L96 lies at the bottom of the antigen-binding pocket, suggesting that electrostatic interactions between antibody and antigen are important for strong binding (1). The position of the Arg L96 side chain was used to orient antigen and substrate in the binding pocket. Complexes with bound antigen (1) and substrate (2) were constructed and refined by constrained energy minimization. The importance of the Arg L96 side chain was tested by site-directed mutagenesis in the Benkovic laboratory. A mutant with Arg L96 replaced by Gln continues to bind both major fragments of the substrate, but showed decreased antigen binding and no catalytic activity, as predicted by the model.

The model indicates that, even though the NPN43C9 antibody has two His residues near the binding site pocket, they are too far apart to simultaneously bind a metal ion. Consistent with this, fluorescence quenching studies indicated that the single-chain construct of the NPN43C9 antibody had no detectable binding affinity for the metal ions Cu(II), Zn(II), Co(II), or Fe(II) (3). By transplanting metal-binding templates from metalloenzymes into the antibody structural model, we identified potential metal-binding sites within the antigen-binding pocket, where bound metal could be detected by spectral changes of the antibody or of bound antigen analogs.

A Metal-Binding Site with the Metal Bound at the Bottom of the Binding Pocket.
Placing a metal ion at the bottom of the antigen-binding pocket will allow substrate-metal interactions, providing potential probes for metal binding. Superposition of the Zn-binding ligands from carboxypeptidase, superoxide dismutase, and thermolysin onto the coordinates of the NPN43C9 antibody model indicated that the tetrahedral arrangement of the side chains of residues L91, L96, and H95 resembled the geometry of a metal-binding site

(4). Appropriate mutation of these residues to metal-binding ligands would position the metal ion at the bottom of the antibody binding site.

Replacement of Arg L96 by His should create a two-His metal-binding site with naturally occurring His L91. The model indicated that a metal ion bound by His L91 and L96 would be about 2 Å from the Tyr H95 hydroxyl group, which might therefore act as a third metal ligand. Replacement of Tyr H95 by His could provide a third His ligand for metal binding.

In the Benkovic laboratory, these two mutants were made and characterized by pulsed EPR experiments and UV-visible spectroscopy (5), confirmed the number and type of metal-ligating residues predicted by the model. The Arg L96 to His mutant has two His ligands and a Tyr hydroxyl bound to Cu(II), and the double mutant has three His ligands to Cu(II). Both mutants favor Cu(II) binding over Zn(II), with dissociation constants of 0.5 μ M for Cu(II) and 10 μ M for Zn(II) for the single-site mutant (Arg L96 to His) and 2.1 μ M for Cu(II) and 10 μ M for Zn(II) for the double-site mutant (Arg L96 to His, Tyr H95 to His). These mutants follow the Irving-William series for the order of metal specificity found in model compounds involving Lewis acid catalysis by doubly charged metal ions: Cu(II) is favored over Zn(II) as well as Cu(II) > Ni(II) > Co(II) > Fe(II) > Mn(II). This expected preference was previously seen in metal-binding mutants of the 4-4-20 antifluorescein antibody (6-8).

Design of NPN43C9 Metal-Binding Sites with Preference for Zn(II). In carbonic anhydrase, the Zn(II)-binding site is formed by three His ligands on two anti-parallel β -strands with main-chain hydrogen bonds between two of the His residues. Two sites in NPN43C9, one with metal-ligating residues solely from the VL domain and one with metal-ligating residues solely from the VH domain matched this template (4). These two sites would position the bound metal ion on opposite sites of the binding pocket. The VL site included replacement of VL residues Tyr L32 and Ala L34 by His and the existing His L91. The VH site included replacement of VH residues Asn H33 and Tyr H95 by His and the existing His H35. In the Benkovic laboratory, these two mutants were made and shown to bind Zn(II) tightly with similar dissociation constants (3.3 μ M for the VL site, 1.5 μ M for the VH site) (3,5). The VH site shows over 100-fold selectivity for Zn(II) over Cu(II), Co(II), Cd(II), Ni(II), and Fe(II) (3). The VL site showed no detectable Cu(II) binding. Thus, these sites are unusual in that they do not follow the Irving-William series for metal-ion binding, indicating that the protein environment is influencing metal-ligand geometry.

In addition, both these mutants showed unusual quenching of intrinsic protein fluorescence upon Zn(II) binding. Usually, Cu(II) binding is measured by fluorescence quenching and Zn(II) binding is measured by competition with bound Cu(II), resulting in a return of intrinsic protein fluorescence. Fluorescence quenching by Zn(II) is particularly strong in the VH site (about 85%), providing a direct indicator for Zn(II) binding. The unusual binding and spectral qualities shared by these mutants may be due to the structural similarities of the two sites. Framework residue Trp H47, which may be principally responsible for the intrinsic fluorescence of the Fv domain (3) lies immediately under the binding site near residues L34 and H35. The NPN43C9 model shows that the His H35 side chain contacts the indole ring of Trp H47 (2). The unusual fluorescence quenching by Zn(II) may operate by a mechanism similar to that seen in the protein barnase, in which protonation of a solvent-exposed His that contacts a buried Trp residue causes fluorescence quenching (9). The VL and VH domains are

structurally homologous and can be related by rotation about a pseudo-2-fold axis centered between them. This reveals a one-to-one correspondence between the residues in the VL site (L32, L34, and L91) and those in the VH site (H33, H35, and H95) and shows highly similar surrounding protein environments (4). Thus, it is likely that the protein environment in these two mutants imposes the metal selectivity by constraining the ligand geometry in similar ways. The model also predicts that bound Zn(II) in the VH site is near the bound *p*-nitrophenol group of the hapten, suggesting that the two ligands may interact electronically. The Benkovic group has verified this by showing that Zn(II) addition perturbs the absorbance spectrum of bound *p*-nitrophenol (3). This effect is reversed by addition of EDTA.

SIGNIFICANCE: From our high-resolution models of the NPN43C9 catalytic antibody with bound antigen and substrate, we have designed three distinct metal-binding sites. These three sites, one with ligands in the VL domain, one with ligands in the VH heavy domain, and one with ligands in both domains, were made by site-directed mutagenesis and shown spectroscopically to bind metal ions as predicted. These results demonstrate the success of our design approach, the versatility of the antibody structure for metalloprotein design, and the validity of the three-dimensional model. One site shows selectivity for Cu(II) over Zn(II), while the other two sites display a more unusual preference for Zn(II) over Cu(II). Thus, we have designed metal-binding sites having different metal specificities into a single antibody. A panel of such mutants proteins, based on a single antibody, could act as a biosensor for multiple metal ions. Metal binding can be measured directly by examining changes in intrinsic protein fluorescence or indirectly by changes in spectral properties of simultaneously bound antigen analogs.

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Design of Metal-Detecting Antibodies

Objectives

Build a model of the NPN43C9 antibody for directing the design of potential metal-binding sites.

Characterize metal-binding sites in proteins with known three-dimensional structure and test these metal-binding templates by transplanting them into the NPN43C9 antibody.

Accomplishments

Refinement of the NPN43C9 antibody model, including docked antigen and substrate. Identification of conserved side chain features of antibody structure.

Design of two metal-ion-binding sites, one in the light chain and one in the heavy chain, based on the Zn(II) site template from carbonic anhydrase. Construction and characterization of the sites revealed that both show an unusual preference for binding Zn(II) over Cu(II).

Design of a metal-ion-binding site with the metal ion centered at the bottom of the binding pocket. This two-His site binds Cu(II) preferentially over Zn(II).

Significance

Our approach to modeling antibody structure provides models that are sufficiently accurate to predict mutations for creating metal-binding sites.

Metal-binding sites with differing metal ion selectivities can be built into antibody molecules.